(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 264 893 A1

4

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
11.12.2002 Bulletin 2002/50

(21) Application number: **01113962.3**

(22) Date of filing: **08.06.2001**

(51) Int Cl.[7]: **C12P 7/64**, A61K 35/74, A23L 1/03, A23K 1/00

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(71) Applicant: **Teagasc Dairy Products Research Centre
Cork (IE)**

(72) Inventor: **The designation of the inventor has not yet been filed**

(74) Representative: **Dörper, Thomas Michael
BASF Aktiengesellschaft,
ZDX - C6,
Abteilung 'Patente,
Marken und Lizenzen'
67056 Ludwigshafen (DE)**

(54) CLA biosynthesis by bifidobacteria

(57) Nineteen strains of *Lactobacillus*, 2 strains of *Lactococcus*, 1 strain of *Pediococcus*, 4 strains of *Propionibacterium* and 23 strains of *Bifidobacterium* were screened for their ability to produce conjugated linoleic acid (CLA) from linoleic acid. Of these, 7 strains of *Bifidobacterium*, as well as 2 strains of *Propionibacterium* produced the *cis-9*, *trans*-11 CLA isomer from linoleic acid. In contrast, strains of *Lactobacillus, Lactococcus* and *Pediococcus* lacked the ability to synthesise CLA. CLA (*cis*-9, *trans*-11 isomer) production by the genus *Bifidobacterium* was shown to exhibit considerable interspecies variation, with *B. breve* and *B. dentium* being the most efficient producers among the strains tested, yielding up to 65 % conversion of linoleic acid to CLA at linoleic acid concentrations of 0.2-1.0 mg/ml in MRS medium. The growth of *B. breve* strains was inhibited by increasing concentrations of linoleic acid. Viability of *B. breve* 2257 was unaffected in the presence of up to 0.5 mg/ml linoleic acid for 48 h but was dramatically reduced to 1.5% survival at 1 mg/ml linoleic acid. However, viability of the *B. breve* strains NCFB2258, NCTC 11815, NCIMB 8815 and NCIMB 8807 was reduced to < 60 % at linoleic acid concentrations of 0.2 mg/ml. These data suggest that certain strains of bifidobacteria may have applications to elevate CLA content of food products and CLA status in humans.

EP 1 264 893 A1

Printed by Jouve, 75001 PARIS (FR)

BEST AVAILABLE COPY

Description

INTRODUCTION

[0001] Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid, and has gained considerable attention in recent years because of the many beneficial effects attributed to the *cis*-9, *trans*-11 and trans-10, cis-12 isomers, in particular. These include anticarcinogenic activity, antiatherogenic activity, the ability to reduce the catabolic effects of immune stimulation, the ability to enhance growth promotion and the ability to reduce body fat (Martin and Banni, 1998 for review, and references therein). Of the individual isomers of CLA, *cis*-9, trans-11-octadecadienoic acid has been implicated as the most biologically active because it is the predominant isomer incorporated into the phospholipids of cell membranes, liver phospholipids and triglycerides (Kramer *et al.*, 1998). This isomer is also the predominant dietary form of CLA, obtained from fats derived from ruminant animals, including milk, dairy products and meat (Chin *et al.*, 1992, O'Shea *et al.*, 2000).

[0002] In addition to the ability of certain rumen-derived strains, including *Butyrivibrio fibrisolvens* to form CLA from dietary linoleic acid (Kepler and Tove, 1967), it has also been shown that certain cultures used in food fermentations possess the ability to generate *cis*-9, *trans*-11 CLA. Strains of the intestinal flora in rats (Chin et al., 1994), two strains of *Propionibacterium freudenreichii* spp.. *freudenreichii* and one strain of *P. freudenreichii* subsp. *shermanii* (Jiang *et al.*, 1998), and six lactic cultures, including *L. acidophilus* (Lin *et al.*, 1999) have been shown to possess this capability. In this study, we assessed a collection of strains, many which are human intestinal isolates (previously isolated from the human GIT) with probiotic potential, for ability to form the *cis*-9, *trans*-11 CLA isomer, using linoleic acid as the substrate.

MATERIALS AND METHODS

Maintenance of bacterial strains

[0003] The 19 strains of *Lactobacillus*, 2 strains of *Lactococcus*, 1 strain of *Pediococcus*, 4 strains of *Propionibacterium* and 19 strains of *Bifidobacterium* used in this study are listed in Table 1. The UCC strains used in the study were previously isolated from the human gastrointestinal tract (GIT) and obtained from University College Cork, Ireland under a restricted materials transfer agreement.

[0004] The *Lactobacilli, Pediococci* and *Bifidobacterium* strains were cultured in MRS (Difco Laboratories, Detroit, MI, USA) under anaerobic conditions (anaerobic jars with 'Anaerocult A' gas packs; Merck, Darmstadt, Germany) and 1.5 % (w/v) agar (Oxoid Ltd. Basingstoke, Hampshire, UK) was included for plating. *Pediococci, Lb.reuteri* NCIMB 702655, *Lb. reuteri* NCIMB 7025656 and *Lb. reuteri* DSM 20016 were routinely cultured at 30°C and the remaining *Lactobacillus* strains were cultured at 37°C for 24 h. For *Bifidobacterium,* 0.05 % (w/v) L-cysteine hydrochloride (98 % pure, Sigma Chemical Co. St. Louis, MO, USA) was added to the medium and cultures were grown for 48 h at 37°C under anaerobic conditions. *Lactococcus* strains were cultured in MRS under aerobic conditions at 30°C for 24 h. The *Propionibacterium* strains were cultured in sodium lactate medium (SLM, Malik *et al.* 1968) at 20°C for 72 h under anaerobic conditions. Total viable counts were determined by pour plating of 10-fold serial dilutions in Maximum Recovery Diluent (Oxoid), using MRS agar for lactobacilli and MRS agar with 0.05 % (w/v) cysteine for bifidobacteria.

Assay for microbial CLA production

[0005] Prior to examination of the strains for CLA production, each was subcultured twice in MRS broth (supplemented with cysteine, 0.05 % w/v for *Bifidobacterium*) for 48 h, using a 1 % innoculum. All strains were then cultured in MRS broth (supplemented with cysteine, 0.05 % w/v for *Bifidobacterium*), spiked with different concentrations of free linoleic acid (LA: *cis*-9, *cis*-12-octadecadienoic acid, 99 % pure, Sigma Chemical Co.). This was added as a 30 mg/ml stock solution of linoleic acid in 2 % (v/v) Tween 80 (polyoxyethylene sorbitan mono-oleate; Merck-Schuchardt, Germany), which was previously sterile-filtered through a 0.45 μm Minisart filter (Sartφrius AG, Germany). The strains were inoculated to a density of 10^6 cfu/ml in free linoleic acid-containing MRS media and incubated for their respective times and temperatures (described above). Following incubation, 5 ml of the cultures were centrifuged at 960 × g for 5 min at room temperature (Sanyo MISTRAL 2000R Centrifuge).

[0006] The fatty acid composition of the resulting supernatant was analysed as follows. Initially, $C_{13:0}$ (tridecanoic acid, 99 % pure, Sigma Chemical Co.) was added to 4 ml of the resulting supernatant, as an internal standard at a concentration of 0.25 x the initial linoleic acid concentration and lipid extraction was performed as follows. Two milliliters of isopropanol (99 % purity, Alkem Chemicals Ltd., Cork, Ireland) was added to the supernatant and the samples were vortexed for 30 sec. A total of 4.5 ml hexane (99 % purity, LabScan Ltd., Dublin, Ireland) was added to this and the mixture plased on a shaking platform for 3 min before centrifugation at 960 × g for 5 min at room temperature. The

supernatant (the hexane layer containing the lipids) was removed and the procedure was repeated twice. The hexane layers were pooled and stored at -20 °C prior to preparation of fatty acid methyl esters (FAME) for gas liquid chromatographic (GLC) analysis.

Preparation of Fatty Acid Methyl Esters (FAME) and GLC Analysis

[0007] The lipid extracts in hexane were analysed by GLC following acid-catalyzed methylation as described previously (Stanton *et al.*, 1997). Free fatty acids in oils such as sunflower and soybean oils were calculated as the difference between fatty acid concentrations obtained following acid and base catalyzed methylation, performed using 2 N methanolic KOH (Sigma Chemical Co.) at room temperature.

[0008] The GLC was performed with reference to the internal standard $C_{13:0}$. Separation of the FAME was performed on a Chrompack CP Sil 88 column (Chrompack, Middleburg, The Netherlands, 100 m x 0.25 mm i.d., 0.20 µm film thickness), using helium as carrier gas at a pressure of 37 psi. The injector temperature was held isothermally at 225°C for 10 min and the detector temperature was 250°C. The column oven was held at an initial temperature of 140°C for 8 min and then programmed at an increase of 8.5°C/min to a final temperature of 200°C, which was held for 41 min. Collected data were recorded and analyzed on a Minichrom PC system (VG Data System, Manchester, UK). The *cis*-9, *trans*-11 CLA isomer was identified by retention time with reference to a CLA mix (Nu-Chek- Prep. Inc., Elysian, MN). The percentage conversion to CLA and the remaining linoleic acid in the broth were calculated by dividing the amount of CLA and linoleic acid present in the broth after inoculation and incubation with the various cultures used with the amount of linoleic acid present in the spiked broth before incubation.

CLA production by *B. Breve* NCFB 2258 using triglyceride bound Linoleic acid as substrate

[0009] *B. breve* NCFB 2258 was further investigated for ability to utilise triglyceride bound linoleic acid as substrate for CLA production. *B. breve* NCFB 2258 was inoculated from a fully grown culture into MRS broth with added cysteine (0.05 %) and trilinolein ($C_{18:2}$, *cis*-9, cis-12, 99 % pure, Sigma Chemical Co.), soybean oil and sunflower oil (purchased from a local grocery store) containing known linoleic acid concentrations. The triglyceride mixtures were sterile-filtered through 0.45 µm Minisart filters and added as 5 mg/ml aqueous solutions in 2.5 % (v/v) Tween 80. Substantial vortexing was required to dissolve the fat particles. The volume of the triglyceride stock solutions added was calculated to give a final concentration of 0.2 mg linoleic acid/ml of broth. *B. breve* 2258 was inoculated into MRS broth in the presence of the triglyceride substrates under anaerobic conditions at 37°C and incubated for 48 h.

RESULTS AND DISCUSSION

CLA production by bacterial strains

[0010] Throughout the screening programme, the two *Propionibacterium* strains, *Propionibacterium freudenreichii* subsp. *freudenreichii Propioni* 6 (PFF-6) and *Propionibacterium freudenreichii* spp. *shermanii* 9093 (PFS), previously reported to synthesise CLA from linoleic acid (Jiang *et al.*, 1998) were used as positive controls. The CLA biosynthetic assay was set up, with the positive controls in SLM broth, using similar incubation conditions as described previously (Jiang et al., 1998). GLC analysis confirmed that the two strains did convert free linoleic acid to the *cis*-9, trans-11 CLA isomer following incubation at 20 ºC for 72 h, using CRM (certified reference material) 164 and CLA standards for fatty acid identification (data not shown). However, the levels of CLA produced by the two strains of *Propionibacterium* were lower than that reported previously by Jiang *et al.* (1998), producing ~ 60 µg/ml of CLA in comparison with 111.8 µg/ml previously reported by Jiang *et al.* (1998), using 0.5 mg/ml linoleic acid as substrate. In addition, we found that the amount of linoleic acid remaining in the media following incubation with the PFS strain was ~ 50 µg/ml, compared with 289.5 µg/ml reported previously (Jiang *et al.*, 1998). The variation in these data may be a result of differences in the numbers of cells present during incubation, and possibly as a result of the different procedures used for fatty acid extraction and methylation.

[0011] Three strains of *Propionibacterium* were then examined for their ability to produce CLA. These were *Propionibacterium acidi propionici* NCFB 5633, *Propionibacterium freudenreichii* spp. *shermanii* LMG 16424 and *Propionibacterium freudenreichii* spp. *shermanii* JS (Laboratorium Visby, Tonder, Denmark). The strains were incubated in the presence of 0.5 mg/ml linoleic acid using the same growth conditions and media as described above.

The two *Propionibacterium shermanii* strains synthesized CLA in MRS media while *Propionibacterium acidi propionici* did not produce any detectable CLA product (Table 1). The amounts of CLA produced by the two *Propionibacterium shermanii* strains (12-14 µg/ml) were low however, compared with 60 mg/ml produced by PFS strain in this study.

Screening of Lactobacilli, Lactococci, and Pediococci for CLA production

[0012] A variety of different strains of lactobacilli, lactococci, and pediococci, obtained from various sources (Table 2) were tested for ability to produce CLA from linoleic acid. These strains included a number of probiotic strains including *Lactobacillus salivarius* UCC 43310, *Lactobacillus salivarius* UCC 43348, *Lactobacillus paracasei* UCC 43338, *Lactobacillus paracasei* UCC 43364, *Lactobacillus paracasei* UCC 42319, five strains of *Lb. reuteri* and the bacteriocin producing *Lactcoccus lactis* DPC 3147 strain (Ryan *et al.*, 1996). The strains were inoculated into MRS to a density of ~10^6 cfu/ml and incubated under respective conditions as described above, in the presence of linoleic acid concentrations of 0.5 to 3.0 mg/ml.

[0013] The ability of the strains to grow in the different linoleic acid concentrations varied considerably. Good growth of all five *Lb. reuterii* strains occurred at linoleic acid concentrations up to 1 mg/ml, while at 3 mg/ml, the growth of strains NCIMB 701359 and NCIMB 70256 was completely inhibited (data not shown). At all linoleic acid concentrations investigated, none of the *Lb. reuteri* strains investigated produced CLA in detectable quantities.
Lactobacillus helveticus NCDO 1244 exhibited no growth in 0.5 mg/ml linoleic acid while *Lactobacillus leichmanii* NCDO 302 showed good growth in the presence of high linoleic acid concetrations (3 mg/ml). However, none of the strains produced CLA from linoleic acid (between 0.5 to 3 mg/ml) under the conditions used.

CLA production from linoleic acid among *Bifidobacterium* strains cultured in MRS

[0014] A variety of bifidobacteria obtained from a number of sources (Table 3) were screened for CLA production. Since free linoleic acid was found to be inhibitory to the growth of bifidobacteria strains, the minimum inhibitory concentration of linoleic acid for the *B. breve* strains was initially determined. This involved inoculation (1 % from grown cultures) of the *Bifidobacterium* strains into MRS containing free linoleic acid concentrations ranging from 0.2 to 1.5 mg/ml and incubation under anaerobic conditions at 37°C for 48 h. The pH of the media remained unchanged following the addition of the linoleic acid substrate in this concentration range at pH ~ 6.1. Viable bifidobacteria were enumerated at time zero and following 48 h incubation in the presence of the linoleic acid substrate. Viability of *B. breve* 2257 was unaffecetd in the presence of linoleic acid at concentrations up to 0.5 mg/ml. However, viability was dramatically reduced at 1.0 mg/ml and only 1.5 % survival was observed. In contrast the survival of strains *B. breve* 2258, 8807, 8815 and 11815 was reduced to <60 % at linoleic acid concentrations of 0.2 mg/ml and higher. The bifidobacteria strains were then screened for CLA production from linoleic acid substrate at a concentration of 0.5 mg/ml, using the incubation conditions described above. A number of the *Bifidobacterium* strains investigated produced CLA following incubation in MRS containing 0.5 mg/ml linoleic acid, and the results from this screening program showed that there was considerable inter-species variation in the ability of bifidobacteria to produce CLA (Table 3).

[0015] All 5 strains of *Bifidobacterium breve* species examined tested positive for CLA production with four of these strains producing more than 60 μg/ml CLA, while strain *B. breve* NCFB 2257 produced 15 μg/ml under these conditions. In addition, *B. dentium* NCFB 2243 was an efficient CLA producer, also yielding >60 μg/mg CLA (Table 3), while *B. pseudocatenulatum* NCIMB 8811 produced >15 μg/ml under the experimental conditions employed. Among the other bifidobacteria species investigated, 3 strains of *B .adolescentis*, 2 strains of *B. longum* and 1 strain each of *B. angulatum*, *B. bifidum* and *B. lactis* were all negative for CLA production (Table 3). The exact role of biohydrogenation in the metabolic environment of the bacterial cell is unclear. In the study by Jiang *et al.* (1998), strains which were able to produce CLA were those inhibited by the presence of free linoleic acid, but a positive correlation between CLA production and tolerance to linoleic acid was observe within the three CLA producing strains of propionibacteria. This suggests that the conversion of linoleic acid to CLA is a detoxification mechanism for the bacterial cell. This is supported by the fact that the antimicrobial activity of fatty acids with double bonds of *cis* configuration is stronger than that of *trans* (Kabara, 1983).

[0016] The most efficient CLA producers were strains *B. breve* 8815 and 2258 at linoleic acid concentration of 1.0 mg/ml. The strains *B. breve* 8815, 2258 and 2257 were present at less than 10^4 cells/ml at the highest linoleic acid concentration (1.5 mg/ml) and were not analysed for CLA conversion. *B. breve* NCFB 2258 converted ~50 % of the added linoleic acid to CLA at 0.2 and 0.5 mg/ml linoleic acid concentrations. The ability of *B. breve* NCFB 2258 strain to utilise triglyceride bound linoleic acid as substrate for CLA production, using trilinolein ($C_{18:2}$, *cis*-9, cis-12), sunflower and soybean oils was also investigated. The *B. breve* NCFB 2258 strain was found to be negative for ability to utilise triglyceride bound linoleic acid as substrate at 0.2 mg/ml linoleic acid for CLA production (data not shown), from trilinolein, sunflower and soybean oils. These data are in agreement with a previous study which showed that of 61 rumen isolates with ability to produce CLA, none utilised triglyceride bound linoleic acid (Fujimoto *et al.*, 1993). In addition, trilinolein did not inhibit the growth of *B. breve* 2258 to the same extent as similar concentrations of free linoleic acid (data not shown). This indicates that linoleic acid in the free fatty acid form is more toxic to bifidobacteria than triglyceride-bound linoleic acid.

Microbial biohydrogenation of unsaturated C 18 fatty acids

[0017] *B. breve* 2258 and *P. freudenreichii shermanii* 9093 were screened for their ability to produce CLA. The strains were incubated in duplicate in the presence of 0.5 mg/ml linoleic acid (LA) and CLA (the pure *cis*-9, trans-11 isomer), respectively, using the same growth conditions and media as described above. In order to compare the fatty acid composition, control cultures without added LA and CLA were also incubated using the same conditions. The propionibacteria strain used in this study was previously reported to synthezise CLA from LA (Jiang *et al.*, 1998) and therefore used as a positive control. However, in this study, that result was not reproducible since the strain was clearly inhibited in the presence of LA and CLA and hence grew very poorly. No CLA production from that strain was therefore detected and the results from the GLC analysis are not presented here.
After separation (by centrifugation) of *B. breve* 2258 cells from the supernatant following incubation for 48 h, followed by lipid extraction and methylation, the fatty acid composition of both the cells (pellets) and the supernatant were analysed using GLC .

Change in supernatant fatty acid composition following incubation of *B. breve* 2258 with 0.5 mg/ml LA

[0018] GLC analysis confirmed that B. *breve* 2258 converted LA to CLA. Of the added 0.5 mg/ml, only 0.27 mg/ml (54 %) remained in the supernatant (Fig. 5), while the remainder (46 %) was converted to other fatty acids, preferentially the *cis*-9, *trans*-11 CLA isomer followed by *cis*-9-C 18:1 (oleic acid) and a peak of unidentified fatty acids, which most likely is another CLA isomer, although confirmation of this requires further study. The amount of *cis*-9, *trans*-11 CLA produced was 0.136 mg/ml, and the unidentified fatty acids accounted for 0.03 mg/ml. The amount of these two fatty acids present in the control supernatant was negligible (Fig. 5) There was also a substantial increase of *cis*-9-C 18:1 (oleic acid) (64.8 % compared with the control supernatant), which indicates that *B. breve* 2258 harbours a CLA reductase enzyme that hydrogenates the *trans*-11 double bond of *cis*-9, *trans*-11 CLA. Compared to the control culture there was 64,8 % more stearic acid in the LA added supernatant. Smaller increases were observed also in the concentrations of *trans*-11 C 18:1 (vaccenic acid) (30.3 %) and C 18:0 (stearic acid) (17.5 %) compared with the control supernatant, suggesting that other hydrogenating enzymes may be involved.

Change in membrane fatty acid composition following incubation of *B. breve* 2258 with 0.5 mg.ml LA

[0019] The fatty acid composition of the membranes from the cultures (pellet) grown in MRS medium with 0.5 mg/ml LA was also analysed and compared with the control cultures (Fig. 9). Results are expressed as mg fatty acids from cells/ml fully grown culture (see section 3.4.2) The fatty acid concentration in the pellets in mg/ml is lower than that of the supernatant and therefore are not directly comparable. Results from the GLC analysis show that CLA was incorporated in the cell membranes, whereas the control culture contained negligible CLA. The *cis*-9, *trans*-11 isomer was the most abundant CLA isomer and accounted for 0.012 mg/ml, which represents 70 % of the total CLA isomers (assuming that the unidentified fatty acid peak was other CLA isomers). The content of the *cis*-9-C 18:1 (oleic acid) was increased (by 271 % compared with controls) in the membranes of *B. breve* 2258 cells incubated in LA (0.5 mg/ml), indicating the presence of a CLA reductase, which was capable of reducing the unsaturated *trans*-11 bond in CLA in *B. breve* 2258. The *trans*-11 C 18:1 (vaccenic acid) content of the cell membranes wes reduced (over 4-fold) in the LA treated cells compared with the control cells. As seen in the supernatant, a small increase of 28 % in C 18:0 (stearic acid) was detected in the membranes of the LA treated cells compared with the controls (Fig. 9).

Change in supernatant fatty acid composition following incubation of *B. breve* 2258 with 0.5 mg/ml *cis*-9, *trans*-11 CLA

[0020] In order to evaluate if *B. breve* 2258 possesses enzymes other than the putative linoleic acid isomerase, involved in the biohydrogenation of linoleic acid, studies were undertaken using *cis*-9, *trans*-11 CLA as the substrate. Strain *B. breve* 2258 was inoculated in MRS containing 0.5 mg/ml of the pure *cis*-9, *trans*-11 CLA isomer (Matreya Inc. PA, USA) and incubated for 24 h at 37°C. Following incubation in the presence CLA (0.5 mg/ml), only 0.32 mg/ml (65 %) remained in the supernatant (Fig. 9) with the remaining 35 % converted to other fatty acids. The most predominantly formed fatty acid formed corresponded to the unidentified peak, observed following incubation with LA and eluted at 43 mins (Figs. 6 and 10). We propose this is another CLA isomer which was present at 0.12 mg/ml (71 % of the *cis*-9, *trans*-11 CLA peak). Oleic acid was also formed in the supernatant by *B. breve* 2258 following incubation with *cis*-9, *trans*-11 CLA with an increase of 85.5 % compared with the control supernatant. Smaller increases were also observed in the concentration of *trans*-11-C 18:1 (vaccenic acid) (74.5 % compared to control supernatant) and C 18:0 (stearic acid) (23.9 % compared to control supernatant).

Change in membrane fatty acid compositio following incubation os *B. breve* 2258 with 0.5 mg/ml *cis*-9, *trans*-11 CLA

[0021] The lipid composition of the membrane following incubation of *B. breve* 2258 in *cis*-9, trans-11 CLA was also compared with control cells incubated in the absence of CLA.
The fatty acid composition of the membranes from cultures inoculated in MRS containing 0.5 mg/ml of the pure *cis*-9, *trans*-11 CLA isomer shows that the membrane composition changed compared with the control. *Cis*-9, *trans*-11 CLA was incorporated into the membrane of the culture grown in the presence of CLA (0.03 mg/ml) compared with the culture, grown in the absence of CLA, which contained no CLA (Fig. 12.). The same unidentified fatty acid as observed in the supernatant was also present in the membrane following incubation with *cis*-9, *trans*-11 CLA (0.012 mg/ml). Verification of the identity of this fatty acid will be important, as clearly the bacterial cell has the capacity to convert *cis*-9, *trans*-11 CLA to this compound. the possibility is that it is another CLA isomer, although this was not verified in this study, nor was its exact identity confirmed. This could be accomplished using co-eluting fatty acid standards analyzed by GLC or by GCMS. An increase of 54.8 % in the *cis-9-C* 18:1 oleic acid membrane content was obtained following incubation of *B. breve* 2258 in CLA (0.5 mg/ml). This amount of oleic acid in the membranes, formed relative to the control, was greater in the LA treated cells (2.7-fold increase) than the CLA treated cells. As observed in the cell membranes obtained following incubation of *B. breve* 2258 in LA (0.5 mg/ml), the *trans*-11-C 18:1 vaccenic acid content of membranes was lower in the cells incubated with *cis*-9, *trans*-11 CLA than the control pellets (5.8-fold greater in the control). Only a very small increase was obtained in the content of C 18:0 stearic acid in the membranes of CLA treated cells compared with controls.
[0022] The GLC analysis confirmed that *B. breve* 2258 converted LA to CLA and that a significant amount of another, as yet, unidentified isomer was also formed and the data also, it also indicates that a further biohydrogenation of *cis*-9, *trans*-11 CLA to C 18:1 isomers, preferably the *cis*-9-C 18:1 isomer occurs as a result of incubation with *B. breve* 2258 strain. Since also a small increase of C 18:0 was detected in the chromatogram, it is possible that additional enzymes are involved, but whether this activity is significant is unclear. The increase in saturation was obtained in both the supernatant and the bacterial pellets. Also when the pure CLA isomer was incubated with *B. breve* 2258 it was further hydrogenated to more saturated fatty acids, primarily *cis*-9-C 18:1. This may support the theory that incorporation of a *trans* fatty acids instead of *cis*, and saturation or *trans* conversion of *cis* double bonds is a strategy for the bacterial cell to counteract for the increased fluidity that occurs when LA and the *cis*-9, *trans*-11 CLA isomer (which has a *cis* bond) is interfering with the membranes which leads to expansion of membrane, elevation of membrane permeability and impairment of membrane functions (Junker and Ramos, 1999; Weber *et al.*, 1994).
Interestingly in this study, the differences in fatty acid composition when adding LA and CLA respectively, to the supernatant and pellets, is not very significant. When adding the pure *cis*-9, *trans*-11 CLA isomer to the supernatant it is converted to a great extent to other CLA isomer, which is not the case in the LA added supernatant.
[0023] Because of all the beneficial health effects of CLA, the ability of strains of bifidobacteria, natural inhabitants of the intestine, to convert free linoleic acid to CLA can be considered as a novel probiotic trait. Indeed, it is tempting to suggest that the anticarcinogenic activity ascribed to some of these probiotic bacteria could be linked to their ability to produce CLA. Development of probiotic dairy products with elevated CLA levels also provides an exciting opportunity. Exploitation of probiotic bifidobacteria harbouring CLA biosynthetic capabilities offers novel opportunities in the rational design of improved health-promoting functional foods, with the benefits of enriched CLA and probiotic bacteria.

References

[0024]

Banni S., Angioni E., Casu V., Melis M.P., Carta G., Corongiu F.P., Thompson H., Ip C. Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid. *Carcinogenesis* 20 (6): 1019-1024 (1999).

Belury M.A. Conjugated dienoic linoleate: A polyunsaturated fatty acid with unique chemoprotective properties. *Nutrition Reviews* Vol 53, No 4: 83-93 (1995).

Bergey's Manual of Systematic Bcateriology Vol III (1989).

Brock T.D., Madigan M.T., Martinko J.M., Parker J. Biology of Microorganisms, 7th ed. Prentice-Hall International, London, (1994).

Chin S.F., Liu W., Storkson J.M., Ha Y.L. and Pariza M.W. Dietary sources of conjugated dienoic isomeres of

linoleic acid, a newly recognized class of anticarcinogens. *J. Food Comp. and Anal.*5: 185-197 (1992).

Chin Sou F., Storkson Jayne M., Liu W. Albright Karen J., Pariza Michael W. Conjugated linoleic acid (9,11- and 10,12- octadecadienoic acid) is produced by conventional but not germ free rats fed linoleic acid. *J. Nutr.* 124: 694-701 (1993).

Cook Mark E. and Pariza Michael. The role of conjugated linoleic acid (CLA) in health. *Int Dairy journal,* 8: 459-462 (1998).

Cook M.E., Jerome D.L., Pariza M.W. Broilers fed conjugated linolec acid had enhanced bone ash. *Poultry Science* 76: S41 (1997).

Doyle Ellin. Scientific forum explores CLA knowledge. *INFORM* 9:69-73 (1998).

Fritsche J., Mossoba M.M., Yurawecz M.P., *et al.* Conjugated linoleic acid (CLA) isomers in human adipose tissue. *Zeitschrift Lebensmittel. Untersuchung Forscung A-Food Research & Technology* 205:415-418 (1997).

Fritsche Jan and Steinhart Hans. Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake. *Z Leberism Unters Forsch A* 206: 77-82 (1998).

Fritsche Jan and Steinhart Hans. Analysis, occurence and physiological properties of trans fatty acids (TFA) with particular emphasis on conjugated linoleic acid isomers (CLA)- a review. *Lipid,* 6S: 190-210 (1998).

Fujimoto K., Kimoto H., Shishikura M., Endo Y., Ogimoto K. Biohydrogenation of linoleic acid by anaerobic bacteria isolated from rumen. *Biosci. Biotech. Biochem.* 57(6):1026-1027 (1993).

Goldin Barry R. Health benefits of probiotics. *British Journal of Nutrition,* 80: 293-207 (1998).

Ha Y.L., Grimm N.K. and Pariza M.W. Anticarcinogens from fried ground beef: heat altered derivatives of linoleic acid. *Carcinogenesis* 8:1881-1887 (1987).

Ha Y.L., Storkson J, Pariza M.W. Inhibition of benzo(a)pyrene- induced neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.* 50:1097-1101 (1990).

Herbel Barbara K., McGuire Michelle K., McGuire Mark A., Schultz Terry D. Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. *Am. J. Clin. Nutr.* 67: 332-337 (1998).

Huang Y.C., Luedecke L.O., Schultz T.D. Effect of Cheddar-cheese consumption on the plasma conjugated linoleic acid concentration in men. *Nutr. Res.* 14:373-386 (1994).

Hughes Peter E., Hunter William J., Tove Samuel B.. Biohydrogenation of unsaturated fatty acids. *The Journal of Biological Chemistry* Vol.257 No 7: 3643-3649 (1982).

Ip C, Chin S.F, Scimeca J.A, Pariza M.W. Mammary cancer prevention by conjugated dienoic derivatives of linoleic acid. *Cancer Res.* 51:6118-6124 (1991).

Ip C., Singh M., Thompson H.J. Cojugated linoleic acid supresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Res.* 54: 1212-1215 (1994).

Ip C, Scimeca J.A., Thompson H.J. Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer supression. *Nutr. Cancer* 24:241-247 (1995).

Ip C, Jiang C., Thompson H.J. Scimeca J.A. Retention of conjugated linoleic acid in the mammary gland is associated with tumour inhibition during the post- initiation phase of carcinogenesis. *Carcinogenesis* 18:755-759 (1997).

Jiang Jin, Björk Lennart, Fondén Ragne. Production of conjugated linoleic acid by dairy starter cultures. *J.Appl. Microbiol.* 85:95-102 (1998).

Jiang J., Wolk A., Vessby B. Relation between the intake of milk fat and the occurence of conjugated linoleic acid in human adipose tissue. *Am. J. Clin. Nutr.* 70:21-29 (1999).

Kepler C.R., Tove S.B. Linoleate @[12]- *cis* @[11]- *trans-* isomerase. *Methods in enzymology* Vol XIV p.105 (1969).

Kepler C.R., Tucker W.P., Tove S.B. Biohydrogenation of unsaturated fatty acids. *The Journal of Biological Chemistry* Vol.246 No 14: 3612-3620 (1970).

Kepler C.R., Tucker W.P., Tove S.B. Biohydrogenation of unsaturated fatty acids. *The Journal of Biological Chemistry* Vol.246, No 9: 2765-2771 (1971).

Knekt P., Järvinen R., Seppänen R., Pukkala E., Aromaa A. Intake of daily products and risk of brest acncer. *Br. J. Cancer* 73:687-691.

Kun Lee Yuan and Salminen Seppo. The coming of age of probiotics. *Trends in Food Science & Technology, 6:* 241-245 (1995).

Lal D., Narayanan K.M. Effect of lactation number on the polyunsaturated fatty acids and and oxidative stbility of milk fats. *Indian J. Dairy Sci* 37:225-229 (1984).

Lawless F., J.J. Murphy., D Harrington., R.Devery., C. Stanton. Elevation of conjugated *cis*-9, *trans*-11- octadecadienoic acid in bovine milk because of dietary supplementation. *J. Dairy Sci.* 81:3259-3267 (1998).

Lee K.Y., Salminen S. The coming of age of probiotics. *Trends Food Sci. & Tech. &:* 241-245 (1995).

Lee K.N., Kritchevsky D., Pariza M.W. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis* 108:19-25 (1994).

Liew C., Schut H.A.J., Chin S.F., Pariza M.W., Dashwood R.H. Protection of conjugated linoleic acid against 2-amino3-methylimidazo(4,5-f) quinoline induced colon carcinogenesis in the F344 rat-- a study of inhibitory mechanisms. *Carcinogenesis* 16:3037-3043 (1995).

Lin H., Boylston T.D., Chang M.J., Luedecke L.O., Schultz T.D. Survey of the conjugated linoleic acid contenets of dairy products. *J. Dairy Sci.* 78:2358-2365 (1995).

Malik A.C., Reinbold G.W., Vedamuthu E.R. An evaluation of the taxonomy of *Propionibacterium. Canadian J. Microbiology* 14:1185-1191 (1968).

Miller C.C., Park Y., Pariza M.W., Cook M.E. Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection *Biochem Biophys. Res. Commun.* 198:1107-1112 (1994).

Munday John S., Thompson Keith G., James Kerry A.C. Dietary conjugated linoleic acids promote fatty streak formation in the C57BL/6 mouse atherosclerosis model. *Br. J. Nutr.* 81:251-255 (1999).

Naidu A.S., Bidlack W.R., Clemens R.A. Probiotic spectra of lactic acid bacteria (LAB). *Crit. Rev. Food Sci. Nutr.* 39(1):13-126 (1999).

Nicolosi R.J., Rogers E,J., Kritchevsky D., Scimeca J.A.,Huth P.J. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery* 22:266-277 (1997).

Pariza M.W. Animal studies: summary,gaps and future research. *Am. J. Clin. Nutr.* 66:S1539-1540 (1997).

Rudel L.L. Invited commentary. Atherosclerosis and conjugated linoleic acid.. *Br. J. Nutr.* 81:177-179 (1999).

Salminen Irma, Mutanen Marja, Jauhiainen Matti, Aro Antti. Dietary *trans* fatty acids increase conjugated linoleic acid levels in human serum. *Nutritional Biochemistry 9:* 93-98 (1998).

Sanders M.E. Development of consumer probiotics for the US market. *Br. J. Nutr.* 80(4):S213-218 (1998).

Schonberg S., Krokan H.E. The inhibitory effect of conjugated dienoic derivatives (cla) of linoleic acid on the growth of human tumour cell lines is in part due to increased lipid peroxidation. *Anticancer Res.* 15:1241-1246 (1995).

Schultz T.D., Chew B.P., Seaman W.R. Differential stimulatorry and inhibitory responses of human mcf-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture. *Anticancer Res.* 12:2143-2145 (1992).

Sehat Najibullah, Yurawecz Martin P., Roach John A.G., Mossoba Magdi M, Kramer John K.G. and Ku Youh. Silver ion high performance liquid chromatographic separation and identification of conjugated dienoic linoleic acid isomeres. *Lipids* 33: 217-221 (1998).

Shantha N.C., Moody W.G., Tabeidi Z. Conjugated linoleic acid concentrations in dairy products as affected by processing and storage. *J.Food Sci* 60(4):695-697 (1995).

Stanton C., Lawless F., Kjellmer G., Harrington D., Devery R., Conolly J.F., Murphy J:J. Dietary influences on bovine milk *cis*-9, *trans*-11- conjugated linoleic acid content. *J. Food Sci.* 62:1083-1086 (1997).

Tomatori M. Bifidobacteria and their role in human health. *J. Industrial Microbiology* 6:263-268 (1993).

Van Nostrand R., Douglas M., Considine P.E. Scientific Encyclopedia 7th ed. Vol II (1989).

Visonneau S., Cesano A., Tepper S.A., Scimeca J.A., Santoli D., Kritchevsky D. Conjugated linoleic acid supresses the growth of human breast adenocarcinoma cells in scid mice. *Anticancer Res.* 17:969-973 (1997).

Yang X., Pariza M.W. Conjugated linoleic acid (CLA) -producing bacteria: isolation, identification and properties of their linoleic acid isomerases. *IFT Annual Meeting* p.243 (1995).

Table 1

Screening of Propionibacterium strains for CLA production from linoleic acid in MRS media.		
Strain	**Remaining LA (µg/ml)**	**CLA produced (µg/ml)**
P.acidi propionici NCFB 5633	87.5	-
P. freudenreichii spp. *shermanii LMG 16424*	73.0	12.1
P. freudenreichii spp. *shermanii* JS (Visby)	73.0	13.8

Table 2

Strains screened forCLA production		
Species	**Code**	**Source**
Lactobacillus reuteri	NCIMB 11951	Adult intestine
Lactobacillus reuteri	NCIMB 701359	Unknown
Lactobacillus reuteri	NCIMB 701089	Unknown
Lactobacillus reuteri	NCIMB 702655	From rat
Lactobacillus reuteri	NCIMB 702656	From rat
Lactobacillus reuteri	DSM 20016	
Lactobacillus helveticus	NCDO 257	
Lactobacillus helveticus	ATCC 15009	
Lactobacillus helveticus	NCDO 1244	

Table 2 (continued)

Strains screened forCLA production		
Species	**Code**	**Source**
Lactobacillus leichmanii	NCDO 299	
Lactobacillus leichmanii	NCDO 302	
Lactobacillus fermenticum	ATCC 338	
Lactobacillus acidophilus	ATCC 4356	
Lactobacillus paracasei	UCC 43338	Human GIT
Lactobacillus paracasei	UCC 43364	Human GIT
Lactobacillus paracasei	UCC 42319	Human GIT
Lactobacillus salivarius	UCC 43310	Human GIT
Lactobacillus salivarius	UCC 43348	Human GIT
Lactobacillus	DPC 5336	from cracker Barrel
Bifidobacterium breve	NCTC 11815	
Lactcoccus lactis	DPC 3147	
Lactococcus lactis 290P	DPC 152	
Pediococcus pentasescus	FBB 63	

Table 3 Conversion to CLA by *Bifidobacterium* strains cultured in MRS broth containing cysteine spiked with 0.5 mg/ml linoleic acid for 48 h.				
Species prod.	**Strain**	**Source**	**Growth in 0.5mg/ml**	**CLA**
B. adolescentis	NCFB 2204	Adult intestine	+[1]	0
B. adolescentis	NCFB 2230	Adult intestine	0[2]	-[3]
B. adolescentis	NCFB 2231	Adult intestine	+	0[4]
B. angulatum	NCFB 2236	Human faeces	+	0
B. bifidum	NCFB 795	Human milk	+	0
B. breve	NCFB 2257	Infant intestine	+	++
B. breve	NCFB 2258	Infant intestine	+	+++[7]
B. breve	NCTC 11815	Infant intestine	+	+++
B. breve	NCIMB 8815	Nursing stools	+	+++
B. breve	NCIMB 8807	Nursing stools	+	+++
B. dentium	NCFB 2243	Dental carries	+	+++
B. infantis	NCFB 2205	Infant intestine	+	0
B. infantis	NCFB 2256	Infant intestine	+	0
B. lactis	Bb12	Chr. Hansens	+	0
B. longum	BB536	Visby	+	0
Bifidobacterium sp.	UCC 35612	Adult intestine	+	+[7]
Bifidobacterium sp.	UCC 35624	Adult intestine	+	+
Bifidobacterium sp.	UCC 35658	Adult intestine	+	+

1. growth
2. no growth
3. not determined
4. no CLA produced
5. > 5 μg/ml CLA
6. > 15 μg/ml CLA
7. > 60 μg/ml of broth

(continued)

Table 3 Conversion to CLA by *Bifidobacterium* strains cultured in MRS broth containing cysteine spiked with 0.5 mg/ml linoleic acid for 48 h.				
Species prod.	**Strain**	**Source**	**Growth in 0.5mg/ml**	**CLA**
Bifidobacterium sp.	UCC 35675	Adult intestine	+	+
Bifidobacterium sp.	UCC 35687	Adult intestine	+	++
B. pseudocatenulatum	NCIMB 8811	Nursling stools	+	+

Claims

1. Process for production of CLA by cultivating a microorganism of the genus bifidobacterium in the presence of linoleic acid and isolating the formed CLA.

2. Process according to claim 1 wherein the CLA is cis-9, trans-11 octadecadienoic acid.

3. Process according to claims 1-2 wherein the microorganism is selected from the group consisting of bifidobacterium breve, bifidobacterium dentium and bifidobacterium pseudocatenulatum.

4. Process according to claims 1-3 wherein the concentration of linoleic acid in the culture medium is higher than 1 mg/ml.

5. The use of a microorganism of the genus bifidobacterium as a probiotics in food and feed.

6. The use according to claim 5 in order to prevent or reduce the effects of diarrhoea, infections, cancer, antibiotic treatment.

Fig. 5. Fatty acid composition of supernatant following incubation in MRS medium containing 0.5 mg/ml LA with *B. breve* 2258 for 24 h. The control was *B. breve* 2258 incubated in MRS medium alone.

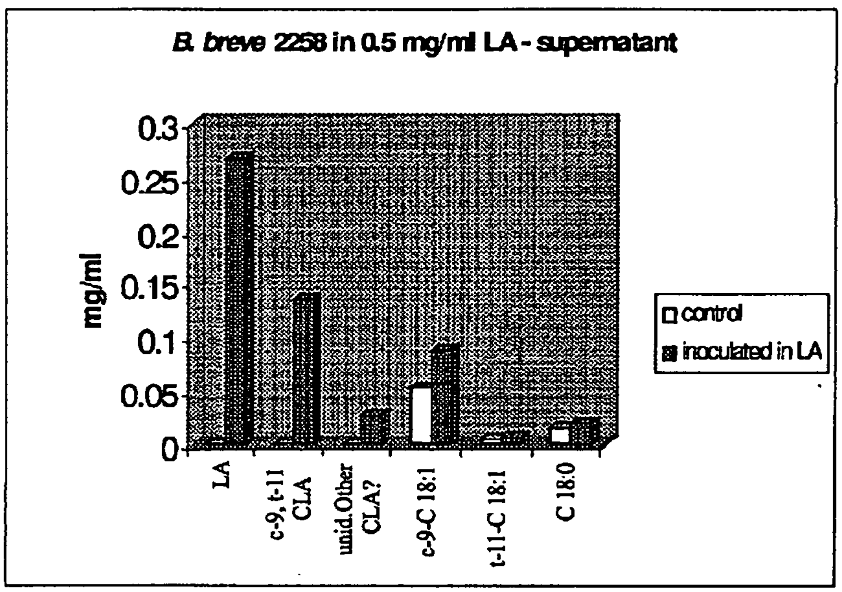


Fig. 6. GLC chromatogram of *B. breve* 2258, control supernatant.

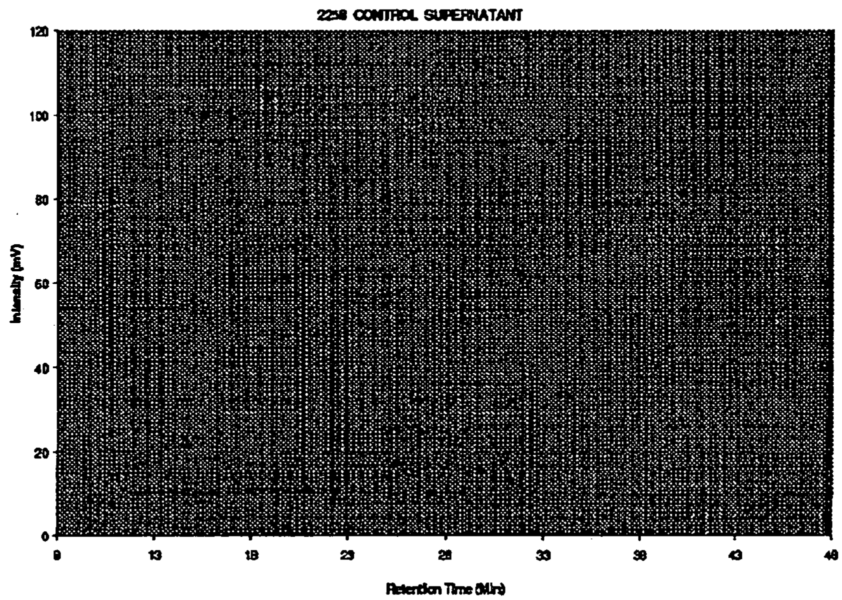


Fig. 7. GLC chromatogram of *B. breve* 2258, added LA supernatant.

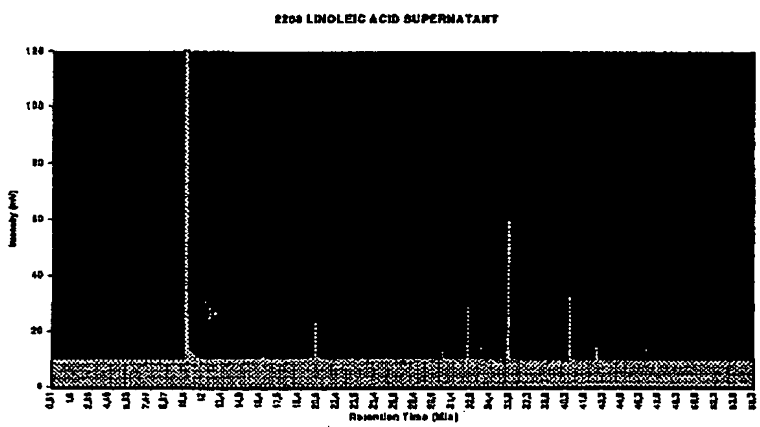


Fig. 8. Chromatogram of CLA standard (Nu- Chek- Prep. Inc. Elysian MN). Separation was performed on Chrompack CP Sil 88 column (Chrompack, Middleburg, The Netherlands) (60 m x 0.25 mm i.d., 0.20 μm film thickness). The retention time on this column is different from the column used for the bacterial fatty acids.

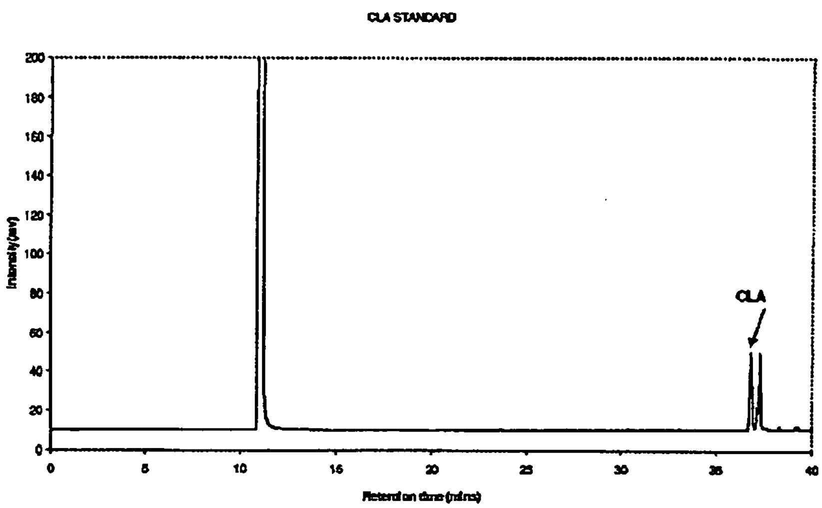


Fig. 9. Fatty acid composition of pellets following incubation in MRS medium containing 0.5 mg/ml LA with *B. breve* 2258 for 24 h. The control was *B. breve* 2258 incubated in MRS medium alone.

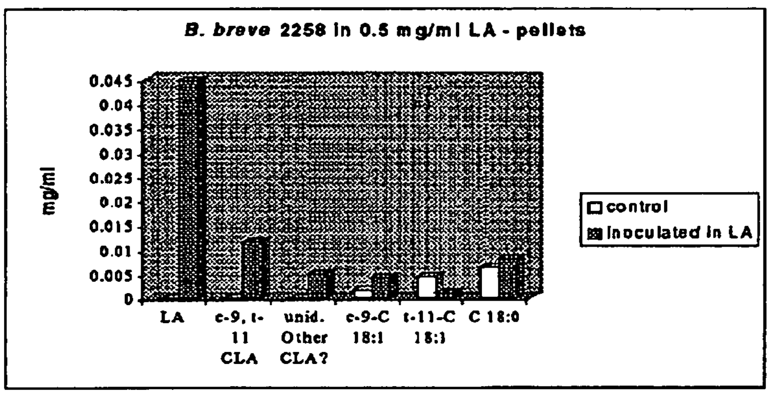


Fig. 10. Fatty acid composition of supernatant following incubation in MRS medium containing 0.5 mg/ml *cis*-9, *trans*-11 CLA with *B. breve* 2258 for 48 h. The control was *B. breve* 2258 incubated in MRS medium alone.

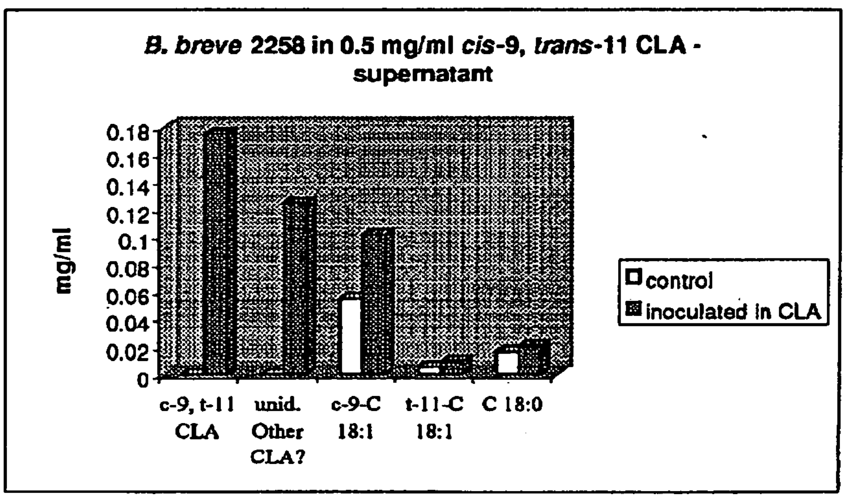


Fig. 11. GLC chromatogram of *B. breve* 2258, added CLA supernatant.

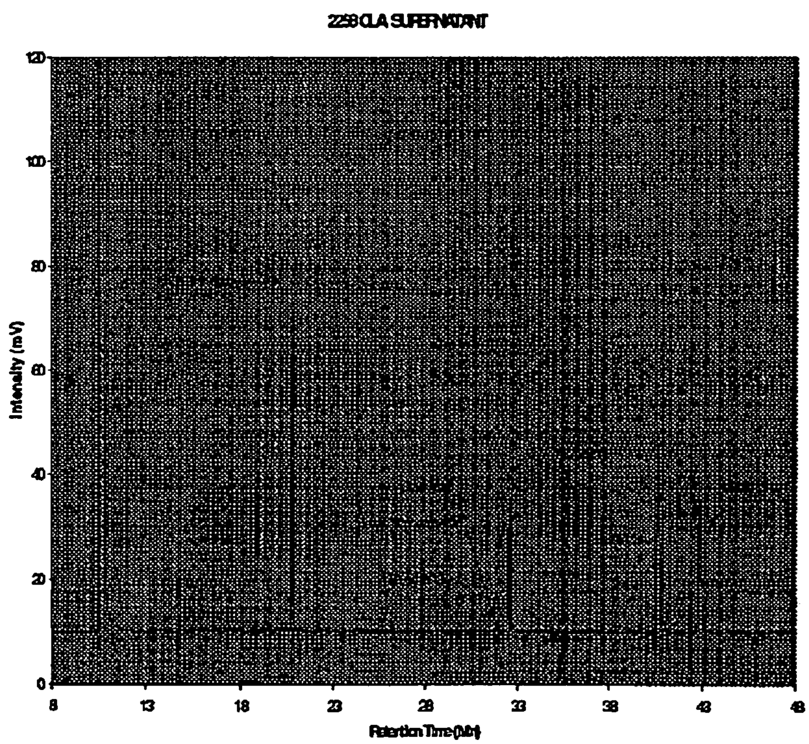
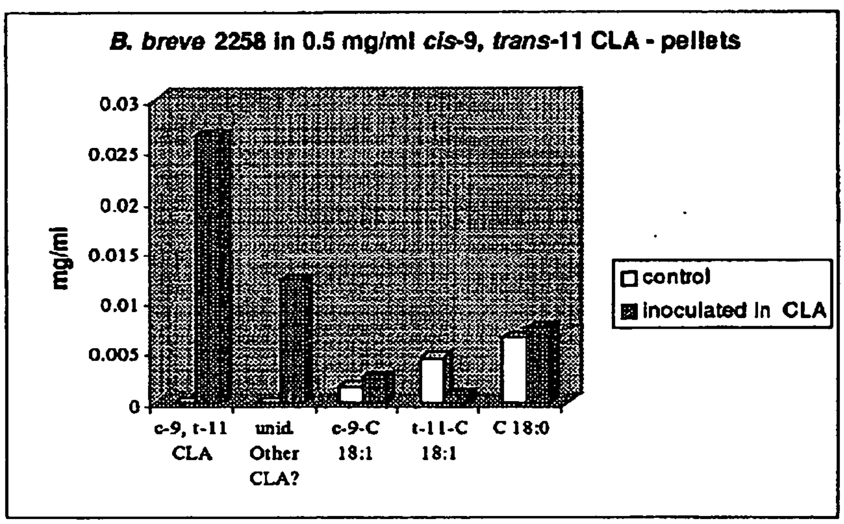


Fig. 12. Fatty acid composition of pellets following incubation in MRS medium containing 0.5 mg/ml *cis*-9, *trans*-11 CLA with *B. breve* 2258 for 48 h. The control was *B. breve* 2258 incubated in MRS medium alone.



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 11 3962

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 99 29886 A (BJÖRK LENNART (SE); FONDÉN RANGNE (SE); JIANG JIN (CN)) 17 June 1999 (1999-06-17) * abstract * * page 10 - page 14 * * page 17; table 1 * * page 23 - page 25; claims *	1-3	C12P7/64 A61K35/74 A23L1/03 A23K1/00

TECHNICAL FIELDS SEARCHED (Int.Cl.7)
C12P
A61K
A23L
A23K

~~The present search report has been drawn up for all claims~~

Place of search	Date of completion of the search	Examiner
THE HAGUE	5 December 2001	Macchia, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

Application Number
EP 01 11 3962

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-4

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 01 11 3962

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-4

   Process for production of CLA.

2. Claims: 5, 6

   The use of a microorganism of the genus Bifidobacterium as a probiotics in food and feed.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 11 3962

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

05-12-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9929886 A	17-06-1999	AU 1792699 A	28-06-1999
		SE 9704584 A	19-07-1999
		WO 9929886 A1	17-06-1999

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: ______________________

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.